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Catalytic Mechanism of Cytochrome P450 for *N*-methylhydroxylation of Nicotine: Reaction Pathways and Regioselectivity of the Enzymatic Nicotine Oxidation

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Abstract

The fundamental reaction mechanism of cytochrome P450 2A6 (CYP2A6)-catalyzed *N*-methylhydroxylation of (*S*)-(–)-nicotine and the free energy profile have been studied by performing pseudobond first-principles quantum mechanical/molecular mechanical (QM/MM) reaction-coordinate calculations. In the CYP2A6-(*S*)-(–)-nicotine binding structures that allow for 5'-hydroxylation, the *N*-methyl group is also sufficiently close to the oxygen of Cpd I for the *N*-methylhydroxylation reaction to occur. It has been demonstrated that the CYP2A6-catalyzed *N*-methylhydroxylation reaction is a concerted process involving a hydrogen-transfer transition state on both the quartet and the doublet states. The *N*-methylhydroxylation reaction proceeds mainly on the doublet state, since the free energy barriers on the doublet state are lower than the corresponding ones on the quartet state. The calculated free energy barriers indicate that (*S*)-(–)-nicotine oxidation catalyzed by CYP2A6 proceeds with a high regioselective abstraction of the hydrogen at the 5'-position, rather than the hydrogen at the *N*-methyl group. The predicted regioselectivity of 93% is in agreement with the most recent experimental reported regioselectivity of 95%. The binding mode of (*S*)-(–)-nicotine in the active site of CYP2A6 is an important determinant for the stereoselectivity of nicotine (*S*)-(–)-oxidation, whereas the regioselectivity of (*S*)-(–)-nicotine oxidation is determined mainly by the free energy barrier difference between the 5'-hydroxylation and *N*-methylhydroxylation reactions.

Introduction

The Tobacco use continues to be the leading global cause of preventable death. It kills nearly 6 million people worldwide each year.¹ Due to the negative health consequences of cigarette smoking, the vast majority of smokers want to quit, but only a few percent of them quit successfully.² The principal addictive compound inhaled in smoking tobacco is nicotine.^{3–4} It is toxic and its metabolism has attracted considerable attention for several decades.^{5–13}

In humans, nicotine is mainly metabolized by the liver, primarily by the liver enzyme cytochrome P450 2A6 (CYP2A6).^{14–18} The principal pathway of nicotine metabolism is initiated by the removal of hydrogen at the prochiral 5'-position on the azaheterocyclic ring of nicotine,^{6, 19} and the product 5'-hydroxynicotine which exists in equilibrium with $\Delta^{1'}(5')$ -iminium ion, is rapidly converted into cotinine by a cytosolic aldehyde oxidase.^{20–22}

Since the two methylene hydrogen atoms at the 5'-position are diastereotopic, this C α oxidation of nicotine may proceed by a process that could result in the selective abstraction of the 5'-hydrogen atom that is *trans* to the pyridine ring (*trans*-5'-hydrogen) or the 5'-hydrogen atom that is *cis* to the pyridine ring (*cis*-5'-hydrogen). Experimental investigations indicated that CYP2A6-catalyzed C α oxidation at the 5'-position of nicotine proceeded with a highly stereoselective loss (89~94%) of the *trans*-5'-hydrogen.^{19, 23} Another CYP2A6-catalyzed metabolic pathway of nicotine is the C α oxidation at the *N*-methyl group of nicotine to form *N*-(hydroxymethyl)nornicotine. *N*-(hydroxymethyl)nornicotine exists in equilibrium with the *N*-methylene-iminium ion⁷ and undergoes spontaneous breakdown to nornicotine and formaldehyde in smokers.^{9, 12} Early experimental studies revealed that CYP2A6 should regioselectively abstract the hydrogen at 5'-position of nicotine, rather than the hydrogen at the *N*-methyl group.^{8, 24-26} The most recent experimental results on CYP2A6-catalyzed nicotine metabolism showed that in the presence of human liver cytosol 95% of the products is cotinine.¹⁸

While the regioselectivity and stereoselectivity of CYP2A6-catalyzed hydrogen abstraction from nicotine (that are 95% and 89~94%, respectively) are high (Scheme 1), the reaction mechanism and the origin of the regioselectivity and stereoselectivity needs to be elucidated theoretically in detail.

In our previous study, QM reaction-coordinate calculations were performed on a simplified model system including nicotine and the active site of cytochrome P450 (Compound I, Cpd I in brief).²⁷ Since the protonated state was dominant for free nicotine in solution (pH 7.4),²⁸ a cationic species of nicotine was used in the QM model system, with the pyrrolidine nitrogen protonated. The protein environment in the QM study was mimicked using a dielectric constant and two N-H...S hydrogen bonds, and the simple model could not account for the actual protein environment of CYP2A6. Most recently, we performed molecular modeling on the (*S*)-(-)-nicotine binding mode at the catalytic site of CYP2A6 enzyme and first-principles quantum mechanical/molecular mechanical (QM/MM) free energy calculations on the CYP2A6-catalyzed 5'-hydroxylation reaction.²⁹ In the QM/MM calculation, the reaction center (the QM region) was treated quantum mechanically while the remaining part (the MM region) of the enzymatic reaction system was treated molecular mechanically. It was demonstrated that the dominant molecular species of (*S*)-(-)-nicotine in the CYP2A6 active site existed in the free-base state (with two conformations, denoted as SR_t and SR_c for convenience), despite the fact that the protonated state was dominant for the free ligand in solution. The commonly observed high stereoselectivity (*trans*-5'-hydroxylation *vs.* *cis*-5'-hydroxylation) was also analyzed. When we prepared this manuscript, Kwiecien *et al.* published their ONIOM QM/MM study on nicotine 5'-hydroxylation and *N*-methyhydroxylation using a simplified model system.³⁰ Their calculations on the simplified model system suggested that *N*-methyhydroxylation required a much higher energy barrier, ca. 6.5 kcal/mol higher, than the 5'-hydroxylation. Notably, only 77 amino acids were included in their model system while the heme was truncated in order to reduce the number of the atoms in the QM part. Our study accounted for all of 467 amino acids of the protein and all heme atoms were included in the QM region of our QM/MM calculation.

In the present study we carried out first-principles QM/MM calculations to account for the actual protein environment of CYP2A6 and used the free-base state of (*S*)-(-)-nicotine to elucidate the mechanism of nicotine *N*-methyhydroxylation. The high regioselectivity favoring hydrogen abstraction from the 5'-position, rather than the *N*-methyl group, was also discussed.

Computational Methods

Structure Preparation

In our recent study, molecular docking and molecular dynamics (MD) simulations led to dynamically stable CYP2A6-SR_I and CYP2A6-SR_C binding complexes.²⁹ The starting structures of first-principles QM/MM reaction-coordinate calculations in the present study were obtained from the stable MD trajectories of the CYP2A6-(*S*)-(-)-nicotine binding structures. There were two different binding modes for neutral (*S*)-(-)-nicotine binding with CYP2A6, *i.e.*, CYP2A6-SR_I and CYP2A6-SR_C. For each binding mode, a snapshot that was close to the average structure simulated was extracted from the stable trajectory as the starting structure of the QM/MM calculations. Prior to QM/MM calculations, each selected starting structure was energy-minimized for 1000 steps by using the Sander module of the Amber 8 program.³¹ Since we were interested in the reaction center, the water molecules beyond 40 Å of the heme iron were removed, leaving the QM/MM system with 945 water molecules and a total of 10485 atoms for the CYP2A6-SR_I complex and 1023 water molecules and a total of 10719 atoms for the CYP2A6-SR_C complex. As illustrated in Fig. 2A, (*S*)-(-)-nicotine, the porphyrin-iron-oxygen complex, and side chain of Cys439 were defined as the QM region (consisting of 104 atoms). The QM/MM interface was described by a pseudobond approach.^{32–34} The pseudobond first-principles QM/MM approach used in the present study has been demonstrated to be a powerful tool in simulating a variety of enzymes (see recent reports^{35–38} and the references cited therein) and some interesting theoretical predictions^{39–42} have been confirmed favorably by experimental studies.^{41–45}

Minimum-Energy Path Determination

With a reaction-coordinate driving method and an interactive energy-minimization procedure,⁴⁶ the enzyme reaction path was determined by the pseudobond QM/MM calculations, in which the QM calculations were performed with the unrestricted B3LYP functional by using a modified version⁴¹ of Gaussian03⁴⁷ and the MM calculations were performed by using a modified version⁴¹ of the AMBER program.³¹ Normal mode analyses were performed to characterize the reactants, intermediates, transition states, and final products. The basis set of the QM part of the QM/MM calculation during geometry optimization was a mixed basis set (denoted as B1), involving 6-31G* for the iron atom and the six atoms coordinated to the iron atom, and 6-31G basis set for all the other atoms. In addition, the energy was corrected by QM/MM single-point calculations with a larger basis set (denoted as B2) which describes iron by the Wachters+f basis set,^{48–49} and the other atoms by the 6-31+G* basis set. For the MM part, the AMBER ff03 force field was used for the protein, and the general AMBER force field (gaff) was used for nicotine. Throughout the QM/MM calculations, the boundary carbon atoms were treated with improved pseudobond parameters.³² No cutoff for nonbonded interactions was used in the QM/MM calculations. For the QM subsystem, the SCF convergence criterion for geometry optimizations was set to 10⁻⁶ (a.u.). For the MM subsystem, the geometry optimization convergence criterion was the rmsd of energy gradient smaller less than 0.1 kcal/(mol · Å). Atoms within 20 Å of the heme iron were allowed to move while all the other atoms outside this range were frozen in all QM/MM calculations.

Free Energy Perturbation

Each reaction path consisted of 70 to 80 individual points. After the minimum-energy path was determined by the QM/MM calculations, the free energy changes associated with the QM-MM interactions were determined by using the FEP method.⁴⁶ In FEP calculations, sampling of the MM subsystem was carried out with the QM subsystem frozen at different states along the reaction path. The point charges on the frozen QM atoms used in the FEP calculation were those determined by fitting the electrostatic potential (ESP) in the QM part

of the QM/MM calculation. The total free energy difference between the transition state and the reactant was calculated with the same procedure used in our previous work.^{29, 50–51} The FEP calculations enabled us to more reasonably determine relative free energy changes due to the QM-MM interaction. Technically, the final (relative) free energy is the QM part of the QM/MM energy (excluding the Coulombic interaction energy between the point charges of the MM atoms and the ESP charges of the QM atoms) plus the relative free energy change determined by the FEP calculations. In FEP calculations, the time step used was 2 fs, and bond lengths involving hydrogen atoms were constrained. In sampling of the MM subsystem by MD simulations, the temperature was maintained at 298.15 K. Each FEP calculation consisted of 50 ps of equilibration and 300 ps of sampling.

Results and Discussion

Mechanistic Insights from MD Simulations

Previous molecular docking and MD simulations²⁹ have demonstrated that (*S*)-(–)-nicotine uses its free-base state (with two conformations, SR_t and SR_c) to bind with the active site of CYP2A6. In the CYP2A6-SR_t complex, the *trans*-5'-hydrogen points toward the oxygen of Cpd I. In the CYP2A6-SR_c complex, the *cis*-5'-hydrogen points toward the oxygen of Cpd I. For each complex, the oxygen of Cpd I can abstract a hydrogen from the 5'-position of (*S*)-(–)-nicotine, leading to the *trans*-5'-hydroxylation reaction or the *cis*-5'-hydroxylation reaction. Experimental studies have indicated that hydroxylation occurs at both the 5'-position (*trans*-5' or *cis*-5') and the *N*-methyl position of (*S*)-(–)-nicotine, although regioselectivity for oxidation of the 5'-hydrogen was commonly observed.^{8, 18, 24–26} A closer inspection on the CYP2A6-(*S*)-(–)-nicotine binding structures reveals that the *N*-methyl group of (*S*)-(–)-nicotine is also sufficiently close to the oxygen of Cpd I for hydrogen abstraction from the methyl to occur, leading to the *N*-methylhydroxylation reaction. The distances from the 5'-hydrogen atom (*trans*-5'-hydrogen or *cis*-5'-hydrogen) and the *N*-methylhydrogen to the oxygen of Cpd I for each binding structure are shown in Fig. 1. In the CYP2A6-SR_t complex, the average distance from the *trans*-5'-hydrogen to the oxygen of Cpd I is ~3.1 Å, while the average distance for the *N*-methylhydrogen is ~2.8 Å. Likewise, in the CYP2A6-SR_c complex, the average distance from the *cis*-5'-hydrogen and *N*-methylhydrogen to the oxygen of Cpd I is ~2.5 Å and ~2.9 Å, respectively. In each of these binding complexes, the 5'-hydrogen (*trans*-5'-hydrogen or *cis*-5'-hydrogen) of (*S*)-(–)-nicotine points toward the oxygen of Cpd I for the 5'-hydroxylation reaction. On the other hand, although the average distance of the *N*-methyl hydrogens from the oxygen is a little bit too long for a reaction to take place, the random rotation of the group sometimes brings the hydrogens close enough, allowing one of the *N*-methyl hydrogen to be abstracted by the oxygen of Cpd I.

Fundamental Reaction Pathway

Molecular docking and MD simulations led to dynamically stable CYP2A6-SR_t and CYP2A6-SR_c complex structures. These stable complex structures were chosen as the initial structures to perform the first-principles QM/MM reaction-coordinate calculations. The active species of CYP2A6, Cpd I, involves two degenerated state (*i.e.* quartet and doublet),⁵² thus, the QM/MM calculations were carried out on both the quartet and the doublet states.

As usual for P450 reactions,^{53–60} the *N*-methylhydroxylation mechanism involves two spin states nascent from the degenerate state of Cpd I. QM/MM reaction-coordinate calculations at the B3LYP/B1:AMBER level reveal that the *N*-methylhydroxylation reaction starts with hydrogen-transfer from the *N*-methyl group of (*S*)-(–)-nicotine to the oxygen of Cpd I, leading to a pair of transition states (^{4/2}TS_H). This C–H bond-activation step is then

followed by an O-rebound process, which leads to C–O bond forming, Fe–O bond breaking, and formation of the product *N*-(hydroxymethyl)nornicotine (Scheme 2). The distances between the C and H (R_{C-H}), distance between the H and O (R_{H-O}), distance between the O and Fe (R_{O-Fe}), and distance the C and O (R_{C-O}) reflect the nature of the chemical process of the reaction. Thus, the distances R_{C-H} , R_{H-O} , R_{C-O} , and R_{O-Fe} were chosen to establish the reaction coordinate as $R_{C-H} - R_{H-O} - R_{C-O} + R_{O-Fe}$ for the reaction-coordinate calculations. The optimized geometries of the reactant complexes (RC), transition states (TS), intermediates (IM), and product complexes (PC) are depicted in Figs. 2 and 3.

In the CYP2A6-SR_t complex, the potential energy surface at the B3LYP/B1:AMBER level clearly shows two transition states (TS_H and TS_{reb}). The geometries of the reactant complex, transition states, intermediates, and product complexes were verified by the full geometry optimizations followed by harmonic vibrational frequency calculations at the same QM/MM level (B3LYP/B1:AMBER). (*S*)-(–)-Nicotine and Cpd I initially form the reactant complex, ^{4/2}RC, in which the distance between the *N*-methylhydrogen and the oxygen of Cpd I is 2.54/2.52 Å. In the geometry of the first transition state, ^{4/2}TS_H, the C–H distance is 1.32/1.29 Å, the H–O distance is 1.29/1.32 Å, and the C–H–O angle is 166.0°/162.9°. Thus, the transition state, ^{4/2}TS_H, has a structure associated with hydrogen-transfer with partially broken C–H bond, partially formed O–H bond, and almost linear arrangement of the C–H–O portion. In the geometry of intermediate ^{4/2}IM, where the hydrogen has been transferred to the oxygen of Cpd I, the distance between the hydrogen of the iron-hydroxo complex and the carbon of the (*S*)-(–)-nicotine moiety is 2.97/2.96 Å. It is apparent that, in the intermediate ^{4/2}IM, the (*S*)-(–)-nicotine moiety is still coordinated to the hydroxyl group of the iron-hydroxo complex. The second transition state was located by saddle-point geometry optimization of all geometric variables with no symmetry constraints, followed by a complete vibrational frequency analysis which revealed that there was only one mode with an imaginary frequency which corresponds to the expected mode of the OH group rotation about the Fe–O bond. The C–Fe–O–H dihedral increases from 47.6/32.7° in ^{4/2}IM to 62.7/44.9° in ^{4/2}TS_{reb}. Once the OH group snaps out of the weak OH–C interaction, the C–O bond between the (*S*)-(–)-nicotine moiety and the hydroxyl group of the iron-hydroxo complex gradually forms, and the Fe–O bond gradually breaks. In the product complex ^{4/2}PC, the hydroxyl group is covalently bonded with the carbon atom at the *N*-methyl position of SR_t while the Fe–O bond no longer exists. According to the two transition states given by the QM/MM potential energy surface, *i.e.* TS_H and TS_{reb}, the *N*-methylhydroxylation reaction involves two steps: the hydrogen-transfer one and the O-rebound one. However, the calculated energy barrier (at the B3LYP/B1:AMBER level) for the O-rebound process is less than 0.1 kcal/mol. Such a low energy barrier is eliminated after single-point energy calculations at the B3LYP/B2:AMBER level, the fluctuation of the MM part, and the thermal corrections of the QM subsystem are accounted for (see below).

Unlike the above-discussed *N*-methylhydroxylation reaction in the CYP2A6-SR_t complex where two transition states (TS_H and TS_{reb}) were determined, the potential energy surface which is determined by the QM/MM reaction-coordinate calculations at the B3LYP/B1:AMBER level shows only one transition state (TS_H) for the *N*-methylhydroxylation reaction in the CYP2A6-SR_c complex. In the optimized reactant complexes, ^{4/2}RC, the *N*-methylhydrogen of SR_c points at 2.59/2.58 Å toward the oxygen of Cpd I. The transition state, ^{4/2}TS_H, has structure of partially broken C–H bond ($R_{C-H} = 1.31/1.27$ Å), partially formed H–O bond ($R_{H-O} = 1.30/1.36$ Å), and almost linear arrangement of the C–H–O portion ($\phi_{C-H-O} = 164.0/164.8$). On the QM/MM potential energy surface, no stationary point was found between TS_H and PC.

Free Energy Barriers

Using the QM/MM-optimized geometries at the B3LYP/B1:AMBER level, we carried out QM/MM single-point energy calculations at the B3LYP/B2:AMBER level for each geometry along the minimum-energy path of CYP2A6-catalyzed (*S*)-(-)-nicotine *N*-methylhydroxylation reaction. For each geometry along the minimum-energy path, the ESP charges determined in the QM part of the QM/MM single-point energy calculation were used in subsequent FEP simulations for estimating the free energy changes along the reaction path. Depicted in Fig. 4 are the free energy profiles determined by the QM/MM-FEP calculations including the zero-point and thermal corrections for the QM subsystem.

For the *N*-methylhydroxylation reaction in the CYP2A6-SR_t complex, the rebound transition state TS_{reb} which is characterized at the B3LYP/B1:AMBER level (Fig. 2E) is eliminated after we consider the B3LYP/B2:AMBER single-point energies, the free energy changes of the MM part, and the thermal corrections for the QM subsystem. Thus, the *N*-methylhydroxylation reaction in the CYP2A6-SR_t complex is effectively concerted and the rebound step is barrierless. The free energy barrier with thermal corrections for the QM subsystem is 18.0/15.5 kcal/mol on the quartet/doublet state. In the CYP2A6-SR_c complex, the reaction proceeds in a concerted way, going to the product *N*-(hydroxymethyl)nornicotine without a distinct rebound step. The free energy barrier for the *N*-methylhydroxylation reaction in the CYP2A6-SR_c complex is 19.4/18.0 kcal/mol. In both the CYP2A6-SR_t and CYP2A6-SR_c complex structures, the *N*-methylhydroxylation reaction proceeds mainly in the doublet state, since the free energy barriers on the doublet states are lower than the corresponding one on the quartet state. In the CYP2A6-SR_t complex, the conformation of nicotine is similar to Model E described by Kwiecie *et al.*,³⁰ but the free energy barriers are ~2 kcal/mol lower than those calculated by Kwiecie *et al.* (19.7 and 17.6 kcal/mol for quartet and doublet, respectively). On the other hand, the free energy barriers in the CYP2A6-SR_c complex (19.4 and 18.0 for quartet and doublet states) are in fairly good agreement with those calculated by Kwiecie *et al.*³⁰

Regioselectivity

Previous experimental studies have indicated that nicotine oxidation by P450 proceeds with a high stereoselectivity of 5'-hydroxylation rather than *N*-methylhydroxylation.^{8, 18, 24–26} Our calculated free energy barriers for *N*-methylhydroxylation (15.5 kcal/mol in the CYP2A6-SR_t complex and 18.0 kcal/mol in the CYP2A6-SR_c complex) are indeed higher than previously determined²⁹ barriers for 5'-hydroxylation (14.1 kcal/mol for *trans*-5'-hydroxylation in the CYP2A6-SR_t complex and 14.4 kcal/mol for *cis*-5'-hydroxylation in the CYP2A6-SR_c complex). As we have already discussed in the previous study,²⁹ the dominant CYP2A6-(*S*)-(-)-nicotine binding structure in solution is the CYP2A6-SR_t complex (~95.4%), while the CYP2A6-SR_c complex also has a significant distribution (~4.4%). Based on the known distribution of CYP2A6-SR_t and CYP2A6-SR_c, the phenomenological free energy barrier for 5'-hydroxylation or *N*-methylhydroxylation may be by using the conventional transition-state theory (CTST)⁶¹ as

$$\Delta G = -RT \ln \left[95.4\% \times \exp\left(-\frac{\Delta G_{\text{CYP2A6-SR}_t}}{RT}\right) + 4.4\% \times \exp\left(-\frac{\Delta G_{\text{CYP2A6-SR}_c}}{RT}\right) \right] \quad (1)$$

Thus, the phenomenological free energy barrier for 5'-hydroxylation was calculated to be 14.1 kcal/mol, whereas the free energy barrier for *N*-methylhydroxylation was calculated to be 15.6 kcal/mol. The corresponding reaction rate constant for 5'-hydroxylation or *N*-methylhydroxylation can also be estimated by using the CTST as

$$k = \frac{k_B T}{h} \exp\left(-\frac{\Delta G}{RT}\right) \quad (2)$$

where k_B is the Boltzmann constant, T is the absolute temperature, and h is Planck's constant. Thus, the reaction rate ratio of 5'-hydroxylation to *N*-methylhydroxylation can be estimated as

$$\frac{k_{5'}}{k_N} = \exp\left[-\frac{\Delta G_{5'} - \Delta G_N}{RT}\right] \quad (3)$$

Based on the reaction rate ratio of 5'-hydroxylation to *N*-methylhydroxylation, we can predict that CYP2A6-catalyzed nicotine oxidation proceeds with a regioselectivity of ~93%, favoring 5'-hydroxylation. The predicted regioselectivity is in good agreement with the most recently reported experimental one, 95%.¹⁸ The excellent agreement between the computational and experimental data may suggest that the computational results are reliable. On the other hand, the excellent agreement could also be a coincidence in consideration of the possible computational errors. In our recently reported computational studies^{29, 35–38, 42, 62–63} on other enzymatic reaction mechanisms using the same QM/MM-FE protocol, the average difference between the computational free energy barriers and the corresponding experimental activation free energies is 0.8 kcal/mol. Nevertheless, the possible computational errors for the relative magnitudes of the free energy barriers calculated for different reaction pathways for a same enzyme system are expected to be smaller and, for this reason, our previously calculated overall stereoselectivity (~97%) of CYP2A6-catalyzed nicotine 5'-hydroxylation reaction was also in excellent agreement with the corresponding data (89 to 94%).²⁹ So, even with the possible computational errors in mind, we may safely conclude that the computational results are qualitatively consistent with the experimental data concerning the regioselectivity.

In the previous study, we have indicated that the stereoselectivity of the CYP2A6-catalyzed (*S*)-(-)-nicotine 5'-hydroxylation reaction originates from the slight preference of SR_t over SR_c in solution and the higher binding affinity of SR_t with CYP2A6 as compared to SR_c binding with the enzyme which lead to the predominance of the CYP2A6- SR_t binding structure in solution. The free energy barriers alone cannot predict the stereoselectivity of the 5'-hydroxylation reaction.²⁹ However, in the present study, we can see that the free energies indeed predict a preference of 5'-hydroxylation over *N*-methylhydroxylation. Thus, it is reasonable to conclude that the binding mode of (*S*)-(-)-nicotine at the active site of the CYP2A6 is an important determinant for the stereoselectivity of (*S*)-(-)-nicotine oxidation, while the regioselectivity of the oxidation is determined mainly by the free energy barrier difference between the 5'-hydroxylation and *N*-methylhydroxylation reactions.

Conclusion

The CYP2A6-catalyzed *N*-methylhydroxylation of (*S*)-(-)-nicotine has been studied by using the first-principles QM/MM approach. The detailed reaction mechanism and the regioselectivity of (*S*)-(-)-nicotine oxidation by CYP2A6 have been elucidated. In the CYP2A6-(*S*)-(-)-nicotine binding structures, the 5'-hydrogen (*trans*-5'-hydrogen or *cis*-5'-hydrogen) on the azaheterocyclic ring of (*S*)-(-)-nicotine is oriented pointing to the oxygen of Cpd I, allowing for the 5'-hydroxylation reaction. In addition, the *N*-methyl group is also sufficiently close to the oxygen of Cpd I for hydrogen abstraction from the methyl to occur, leading to the *N*-methylhydroxylation reaction. The QM/MM reaction-coordinate calculations at the B3LYP/B1:AMBER level indicate that, whereas the *N*-

methylhydroxylation reaction profiles in the CYP2A6-SR_t complex exhibit a transition state of the rebound step, the reaction profiles in the CYP2A6-SR_c descend smoothly toward the product *N*-(hydroxymethyl)nornicotine. However, the low energy barrier for the rebound step in the CYP2A6-SR_t complex is eliminated after accounting for the B3LYP/B2:AMBER single-point energies, the fluctuation of the MM part, and the thermal corrections of the QM subsystem. As a result, in both the CYP2A6-SR_t and the CYP2A6-SR_c complex structures, the *N*-methylhydroxylation reaction proceeds in a concerted way, going to the product *N*-(hydroxymethyl)nornicotine with a barrier-free rebound. The transition state of the CYP2A6-catalyzed *N*-methylhydroxylation reaction is a hydrogen transfer process with partially broken C-H bond, partially formed O-H bond, and almost linear arrangement of the C-H-O portion. The reaction proceeds mainly on the doublet state, owing to the lower free energy barriers on the doublet state than those on the quartet state. The calculated free energy barriers for CYP2A6-catalyzed *N*-methylhydroxylation are higher than those for the 5'-hydroxylation, which is in accord with experimental observations that nicotine oxidation by P450 proceeds with a high regioselectivity of 5'-hydroxylation rather than *N*-methylhydroxylation. The calculated free energy barriers in the present study, along with the CYP2A6-SR_t and CYP2A6-SR_c structural distributions in solution, predict an overall regioselectivity of ~93% which is in agreement with the most recent experimentally determined regioselectivity, 95%. The stereoselectivity of nicotine oxidation catalyzed by CYP2A6 can be attributed to the different binding mode of (*S*)-(-)-nicotine in the active site of the CYP2A6, whereas the regioselectivity of the oxidation is determined mainly by the free energy barrier difference between the 5'-hydroxylation and *N*-methylhydroxylation reactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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(a) CYP2A6- SR_t complex **(b) CYP2A6- SR_c complex** **Fig. 1.**

Plots of the key internuclear distances *vs* simulation time in (a) the CYP2A6-SR_t complex and (b) the CYP2A6-SR_c complex. D1 refers to the shortest distance between the hydrogen on the *N*-methyl group and the oxygen of Cpd I, D2 refers to the distance between the *trans*- or *cis*-5'-hydrogen and the oxygen of Cpd I.

Fig. 2.

Key configurations for *N*-methylhydroxylation of nicotine in the CYP2A6-SR_t complex. The geometries were optimized at the QM/MM (B3LYP/B1:AMBER) level. Values outside the parentheses are for the quartet state, whereas the values in parentheses are for the doublet state. RC: reactant complex. TS: transition state. IM: intermediate. PC: product complex. Distances are in Å and angles are in degree.

Fig. 3.

Key configurations for *N*-methylhydroxylation of nicotine in the CYP2A6-SR_c complex. The geometries were optimized at the QM/MM (B3LYP/B1:AMBER) level. Values outside the parentheses are for the quartet state, whereas the values in parentheses are for the doublet state. RC: reactant complex. TS: transition state. PC: product complex. Distances are in Å and angles are in degree.

Fig. 4.

Free energy profile determined by the B3LYP/B2:AMBER QM/MM-FEP calculations at the B3LYP/B2:AMBER level. Values are relative free energies including the zero-point and thermal corrections for the QM subsystem.

Scheme 1.

CYP2A6-catalyzed nicotine C_α oxidation at the 5'-position (*trans*- and *cis*-) and *N*-methyl position. ^aRelative percentage of each product from Ref. 19; ^bRelative percentage of each product from Ref. 23; ^cRelative percentage of each product from Ref. 18.

Scheme 2.

Reaction mechanism of CYP2A6-catalyzed C_α oxidation at the N -methyl position of nicotine.